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Amino Acids in Asclepias Nectar

Steven Edward Sadwick
The College at Brockport

AMINO ACIDS IN *ASCLEPIAS* NECTAR

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Steven Edward Sadwick

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Steven Edward Sadwick
Master's Degree Candidate

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Major Advisor — Date 9 Dec 83

Committee Member — Date 12/9/83

Committee Member — Date 12-9-83

irman, Graduate Committee

Chairman, Dept. Biological Sciences — 12/9/83

DEDICATION

This paper is dedicated to my wife, and my mother, without whom I never would have finished.

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ABSTRACT

Thin Layer Chromatography in a three solvent system was used to determine the amino acid components in the nectar of three milkweed species. The amino acid complements differed in each species, indicating possible use as a chemosystematic character. The differences in the types of nectarivores or pollinators that visited each species. However, where floral color variations were observed in two of the species, no variation was seen in their respective amino acid complements.

INTRODUCTION

Nectar has long been thought of as an aqueous solution of simple sugars, produced by many species of flowering plants. Much research has been done to identify the sugar constituents that appear in various nectars (Percival 1961; Southwick, Loper and Sadwick 1981). Other recent research has led to the discovery of many other components in nectar, such as amino acids, proteins, lipids, anti-oxidants, alkaloids, glycosides, organic acids and various inorganic substances (see Baker and Baker 1975 for a review). Of these nectar constituents, amino acids are thought to play a role in the complex process of plant pollination (Baker and Baker 1975; Heinrich 1975).

Natural selection has putatively led to a reduction in plant costs, i.e., in nectar production, while maximizing pollination efficiency (Heinrich 1975). Some species of plants produce other materials that attract insects preferentially to themselves. Some of these materials have been listed above. Heinrich (1975) and Pyke (1978) say that at the same time that plants are evolving mechanisms for maximizing pollination at least cost, the insects are thought to utilize foraging strategies which optimize energetic benefit while minimizing metabolic cost.

Amino acids were first found in nectar by Ziegler in 1956 (cited in Baker and Baker 1975), but they were little studied until Baker and Baker began extensive studies on the amino acids in floral and extrafloral nectars. Much of their work has dealt with the relationship of the relative amounts of amino acids present to the major types of pollinators that visit the plant, e.g., short tongued bees, long tongued bees, moths, butterflies, etc., (Baker and Baker 1975, 1977; Heinrich 1975), where the different groups of nectarivores seem to prefer particular concentrations of amino acids. From such work there does appear to be a relationship between amino acid concentrations and pollinator type. This may be evidence of a coevolutionary relationship between plant and pollinator. There is selection for plants that produce additional substances in the nectar, and insect pollinators have preferentially visited those plants which produced amino acids in quantities sufficient to fill their nutritional needs, or affect the flavor preferences in those pollinators.

The amino acids may also affect the taste of the nectar, and hence the attractiveness of the nectar to different species of insects. Shiraishi and Kuwabara (1970) have found that amino acids can impart various tastes (sweet, sour and bitter) to the mixtures tasted by humans. Schoonhoven (1969) and Dethier (1971) indicate that some insects possess chemosensory cells which are

sensitive to amino acids and that not all receptor cells respond to, or in the same way to , the same amino acids, or concentrations of amino acids; differing concentrations of amino acids may elicit different responses in the insect.

The specific amino acids may also provide a cue for the insect in maintaining floral specificity. Baker and Baker (1977) have shown that the amino acid composition in nectar is constant for a given plant species, regardless of geographic location and environmental conditions. Since insects may have the capability for distinguishing different amino acids (Schoonhoven 1969), the amino acids in the nectar provide a distinctive cue which some insects may use to maintain floral specificity. The ability to maintain flower specificity is especially important in some social insects, and insects that have closely evolved with a particular plant species. This is a very difficult area of study, since the insect may use many cues to identify the plant, such as floral color, nectar guides, infrared absorbance zones and scent (Rust 1977; Erickson and Garmet 1979).

Baker and Baker (1976) suggest that the constancy of the nectar amino acids may be useful as a chemosystematic character in identifying closely related species. The Bakers have examined species from a large number of families to illustrate that each species has its own unique complement of amino acids. Where they

compare several species of the same genus, they also show that there is a constancy over many of the components. The actual variability occurs with respect to only a few amino acid components. More work needs to be done in this area before much more can be said about the chemosystematic uses of nectar amino acids. It is, therefore, the purpose of this study to examine the nectar amino acids of three milkweeds of the genus Asclepias: A. syriaca, A. incarnata, and A. tuberosa, to evaluate the value of the nectar amino acid complements as a chemosystematic character.

Members of different plant species are usually separated by differing morphological, chemical or ecological characteristics. The morphological and ecological often are the most widely publicized because they are most easily observable and require no special equipment to determine them. In contrast, the chemical systematists utilize differences in specific compounds which may be produced within the plant. Analysis of nectar components utilizes materials which are secreted by the plant. The nectaries and their product, nectar, are important morphologic and ecologic characters in many plants. By studying the morphologic, ecologic and chemosystematic characters of a species, a more thorough knowledge of that species of plant can be gained. In this study, I will examine what is known about these three Asclepias species, as well as determine, through analysis, if the nectar amino acids

of these three species differ from each other and examine possible reasons why.

ASCLEPIAS

Asclepias is a genus of perennial herbs with a rather unusual flowering form and pollination ecology. The milkweeds have their flowers in umbels with petals reflexed, often concealing the calyx. The hoods are conspicuous and are often mistaken for the petals. These structures are formed from the back of the stamens, and are separated from their base by a short column. These hoods are cup-shaped and form a receptacle in which nectar may collect. These species also possess a horn-like structure, which usually originates from the base of the hood (Gleason 1952). The anatomy of the hood and horn are similar and are not themselves associated with the secretion of nectar (Galil and Zeroni 1965).

The stamens are monadelphous and are fused with the stigma, to form the gynostegium, which conceals the ovaries and styles. The pollen is held in waxy sacs, or pollinia, which are connected into pairs by the translator arms and the corpusculum, comprised of the right and left halves of adjacent stamens.

Pollination in Asclepias has recently been described (Macior 1965; Willson et al. 1979), and is briefly described here. As an insect crawls across the flowers or feeds on nectar, its leg may slip into a space between the hoods,

translator arms, and the gynostegium. As the leg is pulled free, the translator arm or corpusculum may attach to the leg. Once in contact with the air, the pollinium apparatus dries, and the pollinia then rotate. This allows the pollinia to be in the proper orientation to be inserted into the stigmatic chamber of another flower. Due to the rather specific orientation the insect must have with respect to the flower and pollinium, only larger nectarivores are capable of the removal of pollinia, transfer to a new plant and achieve pollination in Asclepias (Willson et al. 1979).

The nectar and pollen systems are spatially and physically separated, preventing contact until the pollinium enters the stigmatic chambers (Galil and Zeroni 1965). This separation makes Asclepias ideal for studies of this type as contact between pollen and nectar is virtually impossible. This feature is important, as Gilbert (1972) and Baker and Baker (1975) indicates that nectar may induce the release of free amino acids in pollen, as well as inducing pollen tube growth (leopold and Kriedmann 1975; Esau 1977). Contamination of nectar samples by free pollen released by the plant at the same time as secretion may also occur easily, as observed by this author on Brassica Kaber, Dentaria sp., Sisymbrium altissimum and Barbarea vulgaris (Cruciferae).

METHODS

The method for collection of nectar was that of Southwick, Loper and Sadwick (1981). The method for the analyses of the amino acids was that of Varga and Richards (1973) as adapted for a miniaturized system by Baker and Baker (1976). A description of the method follows.

Collection of Nectar

Nectar was collected during the early morning, usually between 0600-0830 hrs. Eastern daylight time, from plant specimens which had been bagged with a nylon mesh sac the previous evening. The nectar was collected at this time because sufficient volumes of nectar had been produced for analysis between the time of bagging and 0600-0830 hrs. The bagging was found to be necessary to exclude all nectarivores, pollinators as well as nectar robbing insects such as ants, which were sometimes present on the plants during the entire night.

Nectar was collected with either Drummond one-lambda disposable micropipets (64mm length) or Clay Adams Accufill 90 Micropet disposable micropipets (calibrated 1-5 lambda). Collection of A. tuberosa nectar required the use of an aspirator, as the nectar was too viscous to allow collection by capillary action alone. The nectar samples were removed from the micropipets by using an

eyedropper-like apparatus, supplied by Drummond Scientific Co. for this purpose. Nectar samples were then dried on Whatman No. 1 Chromatography paper and stored individually in plastic bags for later analysis. When sample sizes were very small, viz. 0.25λ or less, several drops were concentrated on the same spot. Attempts were made to concentrate at least three microliters of nectar onto each spot.

Laboratory Analysis of Amino Acids

In the laboratory, the spots were cut out of the chromatography paper, and eluted with about 50 microliters of distilled water, adjusted to pH 8.4 with 0.1M sodium bicarbonate ($NaHCO_3$). This was done in 6mm by 50mm disposable culture tubes. The tubes were refrigerated overnight. The solution was then removed to a clean tube. Dansyl Chloride (1-methyl-aminonaphthalene-5-sulfonyl chloride) was prepared fresh for each use by dissolving the dansyl chloride in acetone. The resulting concentration of this mixture was 1mg/ml acetone. The prepared dansyl chloride was then added to a sample, at a volume equal to the sample. The mixture was then allowed to react overnight at room temperature. As the dansylation reaction proceeded, the color of the reaction mixture changed from yellow to clear. The solution was then concentrated or dried in a vacuum dessicator. The dried samples were then stored until ready for use.

The reaction between dansyl chloride and amino acids is characteristic of the reactions of sulfonyl

chlorides and amines (Morrison and Boyd 1975). In this reaction, the amino acid acts as a nucleophile, attacking the electron rich sulfur. A chloride ion is expelled, as well as a hydrogen ion. An example of this reaction follows (see Figure I).

In excess base, this product is a water soluble salt, which upon addiiton of acid is converted into a water insoluble precipitate (Morrison and Boyd 1975; Solomons 1978). A useful property of the salt is that it fluoresces under ultraviolet (UV) light, enabling the identification of the products once separated by the following procedure.

Prior to use, the samples were reconstituted with 5 µl of distilled water. Quantities as small as 0.5-2.0 µl were then spotted on either side of a two-sided 5cm by 5cm polyamide chromatography plate. Samples were usually spotted on both sides of the plates. The samples were applied using a one lambda micropipet which had been drawn out to a point. Controls containing known dansylated amino acids were also spotted onto plates. (See Table I for list of control amino acids and the concentrations used.) In each case, five microliters of the known amino acids were deposited into a separate clean culture tube, after which 50 microliters of the 0.1M $NaHCO_3$ solution were added to each tube. The known amino acids were then treated the same as the nectar samples.

The plates were then run in the following three solvent systems:

Solvent I: Formic acid: water 1.98.5 V:V

Solvent II: Benzene: glacial acetic acid 9:1 V:V

Solvent III: Ethyl acetate: methanol: glacial acetic acid 20:1:1 V:V:V.

Solvent I was allowed to run to the top of the plate. Before drying, the plate was examined under UV light (366mm). One of the byproducts of the dansylation process is dansyl-OH, which appeared bright blue under UV. This spot should be at least one half way up the plate. If it was not, the plate was allowed to run for a few minutes longer. The plates were then dried using a 1200 watt hand held dryer, and examined under UV.

After examination, the plates were run in Solvent II, at a 90 degree angle to Solvent I. The spots were sharpened up by running the plates twice in this solution, drying the plates between runs. The run was stopped when the solvent front reached about four mm from the top, and the second run stopped at about one mm from the top of the plate. After the plates were dried and examined under UV, the positions of the spots were recorded onto tracing paper. The plates were run in Solvent III in the same direction as Solvent II. The third solvent is run to more clearly separate the amino acids that are closely grouped in the area near arginine. This run was stopped when the

solvent front almost reached the top of the plate. The plate was again dried and the positions of the spots recorded onto tracing paper.

Each run took about 5-10 minutes. The plates, when examined under UV light, were illuminated with wavelengths of 366 nm and 254 nm. The shorter wavelengths were used to determine the presence of amino acids at very low concentrations.

The standard solutions containing known amino acids were run prior to the nectar samples. This was done to facilitate comparing data collected in this study with that of the Bakers.

As mentioned above, care was taken to prevent contamination of samples from pollen and also during the analysis to prevent contamination of materials that could affect the results obtained. The following guidelines were followed by this author to prevent possible contamination.

1. Use disposable poly gloves when handling equipment, culture tubes, pipets or transferring solutions.
2. Never reuse any culture tube or pipet.
3. Never handle any culture tube or pipet near (within about one centimeter) of an open end.
4. Keep all solutions covered when not in use.
5. Minimize all contact with the sample solutions.

In testing the degree to which contaminants could potentially affect the results using this method the following procedure was followed:

1. Fourteen 50µl samples of known amino acids were chosen at random, placed into individual culture tubes, coded and were treated as nectar reservoirs.
2. The above listed precautions to prevent contamination were not followed, although cross contamination between samples between samples was not allowed.
3. The samples were spotted onto the plates and run as normal.

RESULTS

Nectar samples were collected from bagged plants in sufficient volume to determine the amino acid composition of the three Asclepias species. The average volume of nectar collected per flower varied from species to species (Table II). A. incarnata generally contained the greatest volume, while A. tuberosa contained the least. Upon visual examination, nectar volume did not seem to be correlated with flower size (A. syriaca - 7.5mm dia., A. incarnata - 4.5mm dia., A. tuberosa - 6.5mm dia.) but did seem to be related to the relative size of the nectar reservoir. That is, the nectar volume seemed to be related to the volume which could be held within the reservoirs formed by the hoods (Figure II). In A. incarnata, and A. syriaca large amounts of nectar would often be produced, overflowing the nectar reservoir, and into the main part of the hood. In contrast, A. tuberosa never produced nectar in volumes which exceeded the top of the nectar reservoir, even when left bagged for 72 hours.

Asclepias incarnata had only a single genus of nectarivore visiting the flowers during the day. Bombus sp. bees were present, at times in rather large numbers (13-23 per plant $\bar{X} \pm 16.1 \pm 4.01$ SD). On A. tuberosa, a beetle (Chauliognathus sp., fam. Cantharidae) and small

ants were the primary nectarivores during the early portion of the day 0630-1000 hrs. The beetles were gradually replaced by Apis mellifera and small solitary bees as the day progressed. The ants remained as nectarivores on the plants during the entire day. Only a single specimen of butterfly, the common skipper, was ever seen on A. tuberosa. This particular butterfly fed almost continuously on nectar from a single plant for thirty-seven minutes, usually feeding in excess of five minutes per cucullus. A variety of insects, pollinators as well as nonpollinators, were observed feeding on the nectar of A. syriaca; honey bees on A. tuberosa (no pollinia were observed on the beetles present on this species, although this does not necessarily rule out their role as possible pollinators): and bumble bees on A. incarnata. All the plants visited in this study were successfully pollinated, as determined by the presence of seed pods on the plants when visited several weeks later.

The ants were active nectar feeders and also acted to deter the feeding of other nectarivores. This was particularly evident on A. tuberosa, where as few as five ants, when present on an umbel of flowers, prevented honey bees from landing on those flowers. The ants had no apparent effect on the beetles also present on A. tuberosa. A. incarnata had no ants present on the flowers, which was probably due to the inability of the ants to live in the damp ground in which A. incarnata grows.

The standard solutions prepared and run in this study did compare with data supplied by I. Baker (personal communication). Some of the spots were shifted slightly, but the relative position of the different amino acid spots remained the same. In testing for the introduction of impurities into the sampling and testing procedures, the results were unclear and variable. The contaminants appearing in the known amino acid samples were not constant between samples. The number of amino acid contaminants varied from zero to five ($\bar{X}$ 2.4$\pm$.10 SD. for fourteen samples). The most common contaminant appeared to be lysine and serine, each appearing in five of fourteen samples. The color of the contaminating serine was brown, whereas the color of the sample serine was turquoise. The remainder of the spots appeared to be the same color as the sample spots.

Whether these contaminant spots were introduced during the sampling procedure or in preparing the amino acids is unknown, as replicate amino acids were run under more careful conditions with similar results. Some spots may fluoresce brightly enough to be seen from the reverse side of the plate, so caution was necessary when examining the plates, or running samples on both sides of the plates.

Discussion

Pollination in these three species of Asclepias is achieved through the offering of nectar as the sole reward. This contrasts with many other species of plant which offer either pollen alone, or nectar and pollen combined, as a reward for pollination. It may be the nutritive value of the food source which maintains the high visitation rates on Asclepias, not solely its caloric value.

Asclepias syriaca, with the greatest variety of nectarivores, also had the greatest number of nectar amino acids present. Of these nectarivores, only honey bees and a butterfly were large enough to achieve pollination. Willson et al (1979) and Bertin and Willson (1980) also indicate that some, perhaps the most effective, pollination takes place at night by moths. Excluding honey bees and other social insects, which collect nectar solely for its caloric value, A. syriaca may be pollinated diurnally by butterflies and nocturnally by moths. These insects feed almost exclusively on nectar, and their adult lives last only a few days.

Gilbert (1972) describes a species of butterfly (Heliconius sp.) which has an adult life span of three months or more. These butterflies soak pollen they have collected with nectar, releasing amino acids and other constituents, and then imbibe the resulting solution.

Whether the amino acids in nectar alone are sufficient to extend an insect's life span, is as yet unknown.

There are specific attributes of the nectars which may have significant impact on whether or not a particular insect will visit a particular plant. The many mechanisms of evolutionary change (mutation, crossing over, as well as other chromosomal phenomena, and the sexual recombination of genes) have, through competition for pollination, led to a remarkable constancy in floral characteristics for a given species of plant. Percival (1961) and Southwick et al. (1981) indicate that the actual sugar composition of nectars is constant, although the ratios of those components may change with environmental conditions, age of the plant, or other known factors (Percival 1961).

Baker and Baker (1976, 1977) and Rust (1977) also indicate this same specificity with respect to amino acids, viz., that each species of plant has its own, though not necessarily unique, specific amino acid complement. Baker and Baker (1975) and Baker (1977) indicate that there are many other non-sugar components in nectar, which may also distinguish a particular nectar from all others even further. The result is that through a combination of factors the floral nectars of each species are most likely unique with respect to all others.

The successful pollination or survival of the plant species may be due to both the evolution of, as

well as, the constancy of those characteristics cited above. Unfortunately, specific data is greatly lacking in this area, as indicated by Southwick et al. (1981), and more data is needed to bring cohesiveness to the area of nectar secretion in plants.

The amino acid complement of each species examined, Asclepias syriaca, A. incarnata and A. tuberosa, is unique and constant for each species over the area sampled (Table III). Lüttge (1977) and Mayberg and Kristen (1982) indicate in the process of nectar secretion, specific products (sugars) will appear in the nectar, regardless of the specific sugars applied to the plant. The specificity of the nectar components indicate the usefulness of nectar analysis in some taxonomic studies, although more data is needed to determine whether these nectar components remain constant over a greater geographic area.

The insect pollinator species coevolving with a plant species may have had a selective effect on the types of nutrients present in the nectar. In Asclepias incarnata and A. tuberosa nectar, the amino acids are fewer in number than in the nectar of A. syriaca. The pollinators of these two species, Bombus sp. on A. incarnata, Apis mellifera and possibly Chauliognathus sp. on A. tuberosa, are able to feed on both nectar and pollen and, therefore, may not be tied closely to nectar for its nutritional value, but most likely for its caloric value

only. In this case, the amino acids are likely to affect the taste of the nectar or the insect's response to the nectar (Dethier 1971) and its consequent attractiveness to nectarivores.

The amino acid complements of each species overlap each other to a considerable degree (see Table III. This data agrees with Baker and Baker (1976) as described above. This overlap is significant in that it illustrates that the three species do share a considerable degree of genetic similarities dealing with a specific physiologic process within the plant. The degree of similarities may aid researchers in determining how closely, or widely separated two or more species are in their evolutionary history.

A number of the amino acids do vary between the three species, resulting in a unique complement for each species. More data is needed to determine if nectar components do remain constant over wide geographic areas, and also to better compare the amino acids and the ecology of the floral nectarivores.

The presence of color variation in A. syriaca and A. tuberosa, while their respective nectar amino acid compositions remain constant, further indicates that this characteristic may be a useful taxonomic character. There is as yet no evidence that the environmental conditions which lead to significant phenotypic changes in plants will also lead to changes in nectar amino acid content.

Sibling species also present a possible problem, but one could expect that enough physiological and genetic differences would have built up, that it is likely that the nectar amino acid complement would also be different between the two sibling species.

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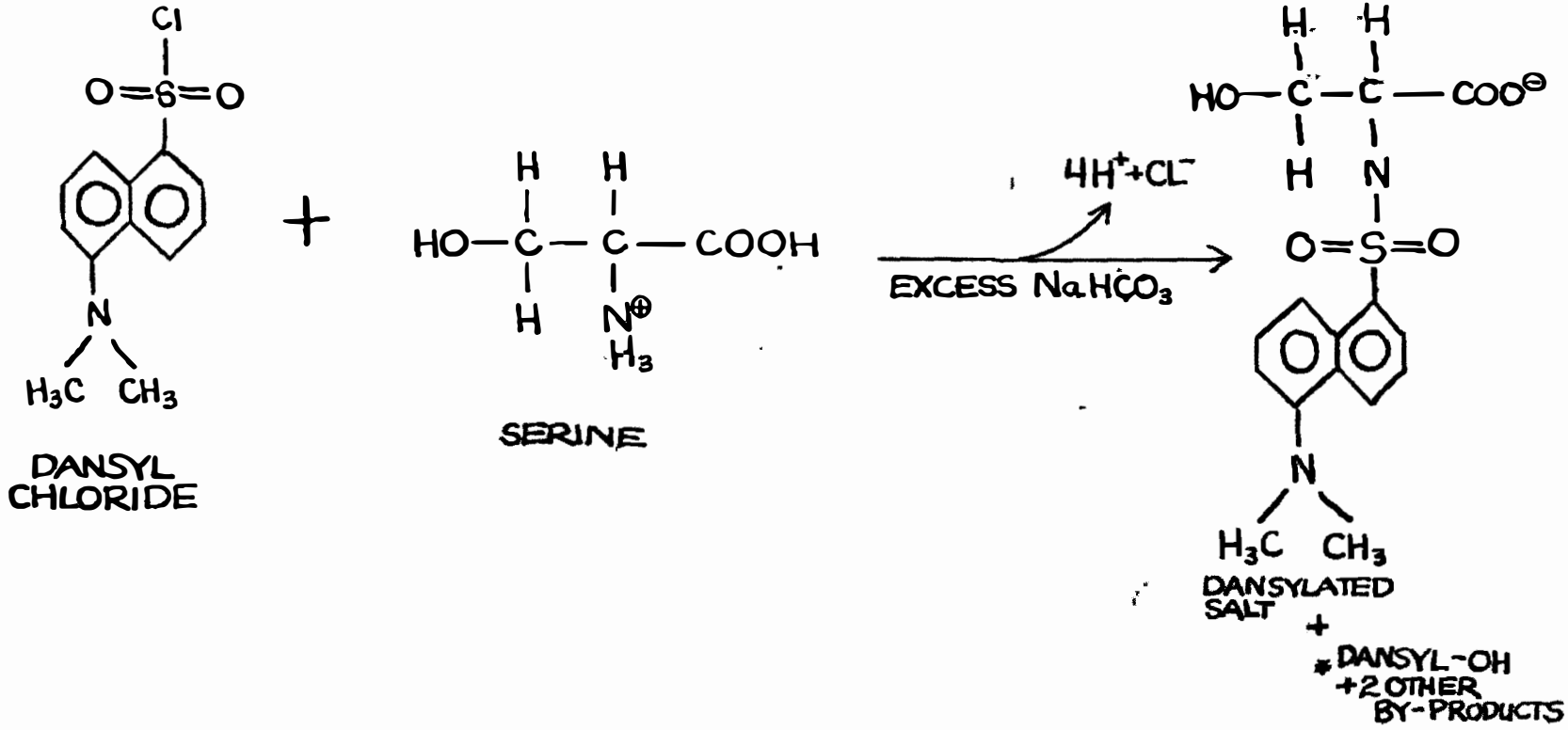

FIGURE I. Typical reaction of an amino acid and dansyl chloride.

*As observed under UV.

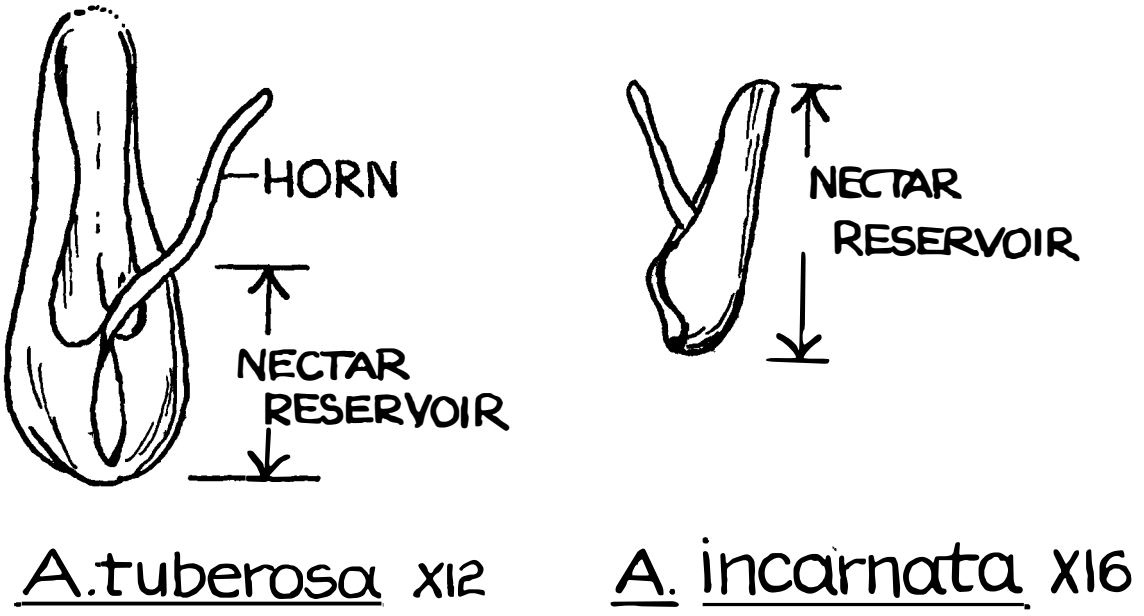

FIGURE II. Hoods of A. tuberosa and A. incarnata depicting nectar reservoirs. Note that the relative sizes of the nectar reservoirs differ with respect to the remainder of the hoods.

(Adapted from Gleason 1952.)

TABLE I

Solutions of known amino acids, utilized in comparisons of known vs sample amino acids and in testing for possible contamination of samples.

Amino acid	Grams	Ml. 20% Sucrose	M x 10^{-4}
Glycine	0.0119	1.0	0.1585
Alanine	0.0119	1.0	0.1336
Valine	0.0119	1.0	0.1015
Leucine	0.0313	3.0	0.0795
Isoleucine	0.0119	1.0	0.0907
Aspartic acid	0.0054	1.5	0.0271
Arginine	0.0102	1.0	0.0484
Lysine	0.0088	1.0	0.0482
Threonine	0.0102	1.0	0.0856
Serine	0.0131	1.0	0.1246
Cystine	0.0146	8.0	0.0076
Cysteine	0.0134	1.0	0.1106
Methionine	0.0150	1.0	0.1005
Phenylalanine	0.0111	1.0	0.0672
Tyrosine	0.0136	4.0	0.0188
Tryptophane	0.0161	1.5	0.0526
Proline	0.0153	1.0	0.1329
Hydroxy-L-Proline	0.0108	1.0	0.0824
Histidine	0.0083	1.0	0.0433
Asparagine	0.0435	2.0	0.1647
Glutamic Acid	0.0098	1.0	0.0692
Glutamine	0.0162	1.0	0.1109

TABLE II

Nectar volumes from bagged plants of *Asclepias syriaca*, *A. incarnata* and *A. tuberosa*.

Species	$\bar{X}$ Vol. $\pm$SD (in microliters)	Range (µl)	No. of Samples with Available Data
A. syriaca	5.71 $\pm$ .10	4-8	14
A. incarnata	4.16 $\pm$ .16	3-5	6
A. tuberosa	1.07 $\pm$ .08	1-3*	10

*Nectar viscosity prevented further nectar removal without causing damage to the flowers, even though there may have been nectar remaining in the flowers.

TABLE III

Amino acid complements of *A. syriaca*, *A. incarnata* and *A. tuberosa*.

Amino acid	*A. syriaca*	*A. incarnata*	*A. tuberosa*
Glycine	+	+	+
Alanine	+	-	+
Valine	+	+	+
Leucine	+	+	+
Isoleucine	+	-	+
Aspartic acid	+	+	+
Arginine	+	+	+
Lysine	+	-	+
Threonine	+	-	-
Serine	+	-	+
Cystine	+	+	+
Cysteine	+	+	+
Methionine	+	+	+
Phenylalanine	+	+	-
Tyrosine	+	-	-
Tryptophane	+	-	+
Proline	+	-	+
Glutamine	+	+	+
Histidine	+	+	+
Asparagine	+	-	-

+ - Present

- - Absent